Endocrine and molecular influences on testicular development in Meishan and White Composite boars

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Abstract

The aim of this study was to evaluate developmental changes in thyroid hormone and other key endocrine hormones/molecular markers produced by testicular cells, in relation to breed differences in proliferation and maturation of Sertoli cells and general testicular morphological development in Meishan (MS) and White Composite (WC) boars. Blood samples and testes were collected on days 60, 75, 90 and 105 post coitum (dpc) and days 1, 7, 14 and 25 post partum (dpp). Testes were immunostained for thyroid hormone receptor-β1 (THRβ1), GATA4, Müllerian-inhibiting substance (MIS), 17-α-hydroxylase ($P450_{c17}$) and inhibin subunits (α, βA, βB). In addition, protein levels were determined by densitometry. Plasma concentrations of free triiodothyronine (T_3) were greater in MS (hyperthyroid) compared with WC (hypothyroid) boars ($P<0{\cdot}01$) during fetal life, but the reverse was evident postnatally. Elevated levels of free T_3 during fetal life were associated with increased levels of THRβ1, suggesting increased thyroid responsiveness of the testis during this time, contrasting with observations during early postnatal life. Localization patterns of THRβ1, MIS, GATA4 and the inhibin subunits were consistent with previous studies. MIS protein levels declined more rapidly ($P<0{\cdot}001$) in MS compared with WC Sertoli cells postnatally, consistent with earlier maturation of Sertoli cells as indicated by our previous study. In this study, transient neonatal hyperthyroidism in MS boars during late gestation was associated with a decline in proliferation and early maturation of Sertoli cells, followed by early onset of puberty in this breed. These observations indicate a possible role for thyroid hormone in the modification of Sertoli cell development, thereby influencing growth and differentiation of the testis in pigs.

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Introduction

A vast array of endocrine and molecular events regulate growth and development; control of testicular development is no exception. Differential timing of Sertoli cell maturation appears to determine the full complement of Sertoli cells in the boar by regulating the period of mitogenesis (McCoard *et al.* 2003 – companion paper). However, the endocrine and/or molecular mechanisms involved in regulation of Sertoli cell maturation and the period of mitogenesis are poorly understood.

Gonadotropins have been implicated in regulating Sertoli cell proliferation (rodents: Davies 1971, Griswold *et al.* 1977, Orth 1984, Meachem *et al.* 1996; monkeys: Marshall & Plant 1996) and Sertoli cell maturation (Griswold 1993). However in boars, plasma follicle-stimulating-hormone (FSH) concentrations are not associated with Sertoli cell proliferation during fetal and neonatal life (McCoard *et al.* 2003 – companion paper), the magnitude of the neonatal increase in FSH is not related to adult testicular size (Ford *et al.* 2001), and the increase in FSH secretion following unilateral castration has minimal effect on Sertoli cell proliferation (Lunstra *et al.* 2003). These observations indicate that magnitude of FSH secretion does not play an important role in establishing the number of Sertoli cells in the boar.

Thyroid hormones also play a role in testicular development. Transient neonatal hypothyroidism delays Sertoli cell maturation in rodents resulting in increased Sertoli cell number, testicular size and sperm production (Cooke & Hess 1992, van Haaster *et al.* 1992, Joyce *et al.* 1993, Bunick *et al.* 1994, De Franca *et al.* 1996). Similar associations between thyroid hormones and testicular development have been observed in rams (Chandrasekhar *et al.* 1985, 1986*a*,*b*, Fallah-Rad *et al.* 2001) and cattle (Majdic *et al.* 1998). Prepubertal 6-N-propyl-2-thiouracil-induced hypothyroidism after 7 days post partum (dpp) does not influence Sertoli cell development in boars

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(Tarn *et al.* 1998). However, goitrogen treatment during periods of maximal Sertoli cell proliferation is critical for maximal responses (Cooke & Hess 1992, Meisami *et al.* 1992), suggesting that induction of hypothyroidism after 7 dpp may be beyond the critical window of adequate 'conditioning' of the Sertoli cell in the boar.

Coupled with the effects on the growth and function of the testis, differential effects on a wide array of hormones and molecular markers are also observed following transient neonatal hypothyroidism. In rodents, these include prolonged early expression of undifferentiated Sertoli cell products, Müllerian-inhibiting substance (MIS) and thyroid hormone receptor (THR), and delayed expression of differentiated Sertoli cell products such as androgen-binding protein, clusterin and inhibin βB (Bunick *et al.* 1994). Differential expression of these important Sertoli cell specific genes are also associated with termination of Sertoli cell proliferation and subsequent maturation in healthy animals (Tran *et al.* 1981, Gondos & Berndtson 1993, Pelliniemi *et al.* 1993). Collectively, these observations support a potential role for thyroid hormone and various Sertoli cell specific products in the regulation of Sertoli cell development. Thus, the aim of this study was to determine whether developmental changes in thyroid hormone and other key endocrine hormones/molecular markers produced by Sertoli cells, are associated with breed differences in proliferation and maturation of Sertoli cells in boars.

Materials and Methods

Sample collection and histological methods

Samples were collected and processed from animals described in the companion paper (McCoard *et al.* 2003 - companion paper). Sections were dried overnight onto glass slides at 37 °C and stained immunohistochemically for GATA4, MIS, thyroid hormone receptor β1 (THRβ1), 17-α-hydroxylase ($P450_{c17}$), and the inhibin subunits (inhibin-α, inhibin βA and inhibin βB) the following day. Immunostaining methods for GATA4 and MIS (McCoard *et al.* 2001*b*) and for $P450_{c17}$ (McCoard *et al.* 2002*b*) have been described previously. The THRβ1 antibody was an anti-THRβ1 peptide antibody (1:200: Santa Cruz Biotechnology, Santa Cruz, CA, USA) raised against a peptide mapping within the amino terminal half of the A/B domain of the thyroid hormone receptor β1 of human origin, as porcine-specific antibodies were not available. This epitope is 85% identical between pigs (CAB42095) and humans (NP_000452). Immunolocalization of THRα1 (Santa Cruz Biotechnology) was attempted but was unsuccessful. This may be due to low abundance below the levels of detection in porcine tissue, or this antibody may not cross-react with porcine tissue. Porcine-specific antibodies were not available. Antibodies directed against the inhibin subunits were mouse monoclonal anti-human peptide antibodies (1:10; Serotec, Oxford, Oxon, UK). The inhibin-α subunit antibody corresponded to residues 1–32 of the 32 kDa α-subunit of human inhibin, inhibin βB subunit corresponded to residues 82–114 of human activin B, and the inhibin βA subunit corresponded to residues 82–114 of the βA subunit of 32 kDa human inhibin A and activin A. Serial sections were also subjected to immunohistochemistry using commercially available peptides (THRβ1-SCB) in 10 times excess of the primary antibodies, or non-immune serum (inhibin antibodies) to confirm the specificity of the antibodies. In addition, absence of the primary antibodies was used to determine non-specific binding.

Slides were deparaffinized in xylene (Sigma; 2 × 5 min) and rehydrated through graded ethanol (2 × 100%, 2 × 95%, 1 × 70%). Antigen retrieval was achieved as previously described (McCoard *et al.* 2001*a*). Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 10 min. Non-specific binding was minimized by incubation for 20 min in 1% normal serum. Sections were incubated with respective primary antibodies for 1 h at room temp (GATA4, MIS, THRβ1, $P450_{c17}$) or overnight at 4 °C (inhibin subunits) in a humid chamber. The avidin-biotin immunoperoxidase system was used to visualize antibody binding (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA). Novared (Vector Labs) was used as the chromagen. The tissue was visualized using light counterstaining with hematoxylin, dehydrated, cleared in xylene and mounted using DPX mounting media (Fluka Biochemica, Steinheim, Germany). For each protein evaluated, all slides were subject to identical staining conditions. Slides were stored at room temperature in the dark until densitometric analysis.

Slides used for breed comparisons within each age group were processed together to ensure each slide was treated identically. The number of slides required to complete breed comparisons for all age groups was substantial and thus all the slides could not be processed within the same assay. Therefore, one testis from a boar at 105 days post coitum (dpc) was selected as intra- and interassay control tissue. One section of this tissue was processed with each assay irrespective of breed, age or protein evaluated, and was used to correct for interassay and intra-assay variation in staining intensity as described below.

Densitometric measurements

Average density measurements in the Bioquant Nova color imaging system (Bioquant Nova 2000 Advanced Image Analysis, R&M Biometrics, Nashville, TN, USA) were used to quantify the amount of protein present for each gene examined using brightfield microscopy as described previously (McCoard *et al.* 2002*b*). Evaluation of staining intensity in Sertoli (THRβ1, MIS, GATA4) and Leydig (THRβ1, $P450_{c17}$) cells was determined by

individually tracing seminiferous tubules and regions of the interstitium containing numerous Leydig cells respectively.

Blood samples: RIA

Plasma thyroid-stimulating hormone (TSH) concentrations were determined by a double antibody RIA (Li *et al.* 1996) that used anti-porcine TSH (AFP 284246) and porcine TSH (AFP10704B) for the reference preparation and for iodination. The coefficients of variation of two pools were 1% and 6%. Plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations were determined with RIA kits that used antibody-coated tubes (DSL, Webster, TX, USA). These assays gave parallel inhibition curves with increasing volumes of pig plasma or serum samples. Coefficients of variation ranged from 2% to 9% for four pools that were included in each T_4 assay and from 2% to 16% for these pools in each T_3 assay. Thyroid binding globulin (TBG) concentration was estimated indirectly by determination of T_3 uptake using T_3-antibody-coated tubes (ICN Pharmaceuticals, Orangeburg, NY, USA). The reference preparation had a mean activity of 34·7% with a coefficient of variation of 4·5%.

For testosterone in plasma, 200 µl were diethyl ether extracted and measured by RIA with antisera supplied by DSL (DSL-4100). Detection of competition was with [^{125}I]-testosterone and the level of sensitivity of the assay was 80 pg/ml. The intra-assay coefficient of variation for testosterone was 8·5%.

Statistical analysis

Differences between breeds in all components estimated were tested using repeated measures in a mixed model procedure (SAS 1999). For fetal samples, the model included fixed effects of breed, age and breed × age interactions and random effects of litter nested within breed. For postnatal samples, the model included fixed effects of breed, age and breed × age interaction, and random effects of litter. For densitometric data, quadrant was the repeated measure. Paired comparisons were made using the Tukey-Kramer procedure. Data were transformed to square roots to adjust for heterogeneity of variance when required. Data are presented as least square means and standard errors (hormone data) or inverse least square means (densitometric data).

Results

Hormone profiles

Triiodothyronine uptake ratio, an estimate of unsaturated TBG binding capacity, increased from 75 to 90 dpc, followed by a decline to 105 dpc in both breeds. A second smaller increase was observed at 1 dpp followed by a steady decline thereafter in both breeds (Fig. 1). WC boars had greater T_3 uptake than MS boars from 90 dpc throughout the remainder of the study indicative of either decreased TBG concentration or greater saturation of normal levels of TBG secondary to thyroid hormone excess compared with MS boars (Fig. 1). The T_3 uptake ratio was used to correct total T_4 and T_3 concentrations providing an estimate of free T_4 and T_3 in the circulation.

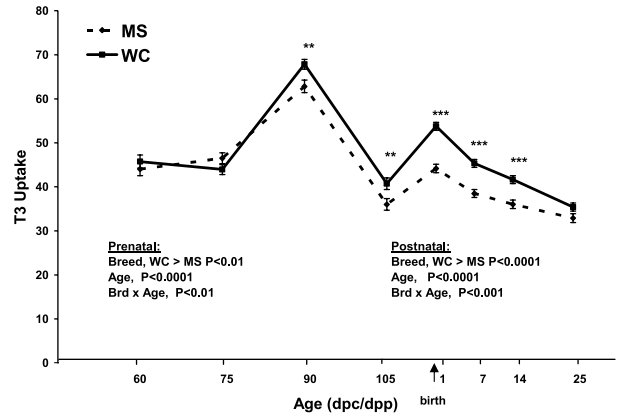

Figure 1 Triiodothyronine (T3) uptake ratio of Meishan (MS) and White Composite (WC) boars during fetal and neonatal life. Data are presented as least square means ± S.E. $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$. dpc, days post coitum; dpp, days post partum; brd, breed.

Plasma concentrations of T_3 increased from 75 to 90 dpc (Fig. 2A). In both breeds, there was a dramatic increase in total T_3 concentrations associated with birth, but total T_3 levels steadily declined thereafter (Fig. 2B). MS boars had 30–40% greater total T_3 levels compared with WC boars during fetal life (Fig. 2A). From 1 to 7 dpp, WC boars had up to twofold greater total T_3 levels compared with MS boars (Fig. 2B). Thereafter, breed differences were not evident. Concentrations of free T_3 in the circulation exhibited similar patterns to total T_3 (Fig. 3A and B). However, a more marked decline in free T_3 was observed from 90 to 105 dpc in both breeds compared with total T_3 (Fig. 3A).

Total T_4 levels increased from 60 to 105 dpc in both breeds, but breed differences were not observed (Fig. 4A). Total T_4 levels declined in both breeds from 105 dpc to 7 dpp, remaining steady thereafter. During early postnatal life, MS boars had greater total T_4 levels compared with WC boars (Fig. 4A). Plasma concentrations of free T_4 increased rapidly during late fetal life until 1 dpp in both breeds, declining thereafter in both breeds (Fig. 4B). Breed differences in free T_4 levels were not observed at any stage.

Fetal profiles of total T_4 and T_3 did not correlate with profiles for TSH during this period of development (Fig. 5). However, elevated free T_3 and T_4 from 105 dpc

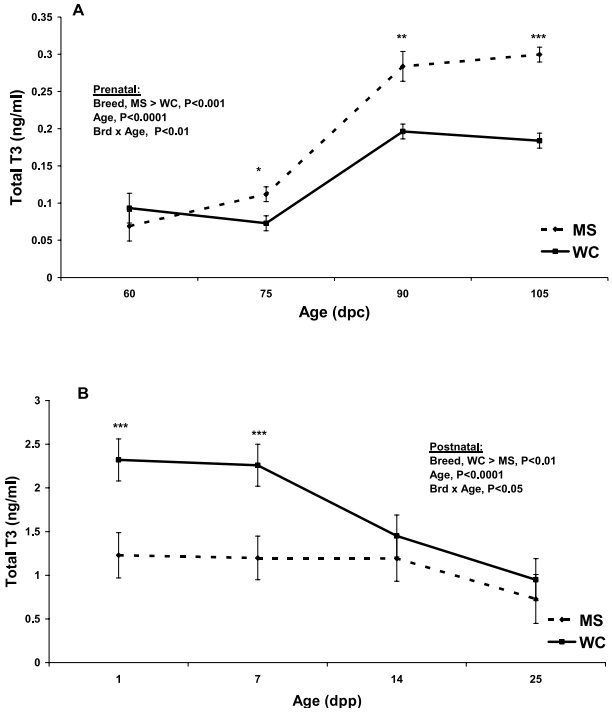

Figure 2 Concentration of total triiodothyronine (T3) in the circulation (ng/ml) of Meishan (MS) and White Composite (WC) boars during fetal (A) and neonatal (B) life. Data are presented as least square means ± S.E. *P<0·05; **P<0·01; ***P<0·001. dpc, days post coitum; dpp, days post partum; brd, breed.

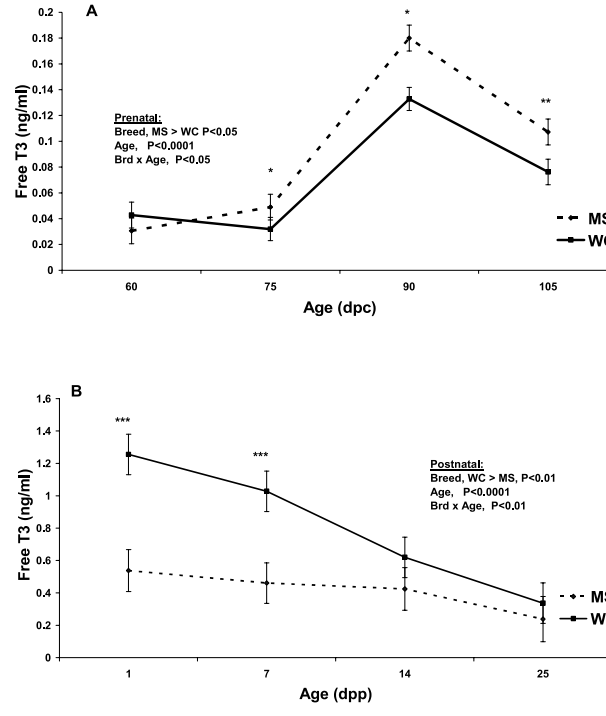

Figure 3 Concentration of free triiodothyronine (T3) in the circulation (ng/ml) of Meishan (MS) and White Composite (WC) boars during fetal (A) and neonatal (B) life. Data are presented as least square means ± S.E. *P<0·05; **P<0·01; ***P<0·001. dpc, days post coitum; dpp, days post partum; brd, breed.

to 1 dpp were correlated with increased plasma TSH during this time, with greater TSH levels in WC boars corresponding to elevated free T_3 and T_4 levels compared with MS boars at 1 dpp (Fig. 5). Whilst TSH levels declined from 1 to 7 dpp remaining constant thereafter in WC boars, paralleling the decline in T_3 and T_4, TSH remained relatively constant during the late neonatal period with a slight increase at 14 dpp in MS boars (Fig. 5).

Testosterone increased with advancing age in both breeds, reaching maximal levels by 14 dpp in both breeds (Fig. 6). Testosterone concentrations were not different between breeds prenatally, but WC boars had greater levels of testosterone compared with MS boars during the early postnatal period (Fig. 6).

Immunolocalization

Thyroid hormone receptor β1 was present in the cytoplasm of Sertoli cells but not in germ cells and also in the cytoplasm and nuclei of Leydig cells (Fig. 7A). THRβ1 protein levels in both Sertoli and Leydig cells increased from 75 to 90 dpc in both breeds followed by a decline until 7 dpp, increasing again thereafter (Fig. 8A,B). Breed differences in Sertoli cell THRβ1 protein levels were not detected, but MS boars tended to have increased Leydig cell THRβ1 protein levels compared with WC boars throughout fetal and early postnatal life. THRβ1 protein levels were up to twofold greater in Leydig cells compared with Sertoli cells throughout the study.

MIS protein was present in the cytoplasm of Sertoli cells throughout development (Fig. 7B). Levels of MIS protein steadily declined during late fetal and neonatal life in both breeds (Fig. 9). Breed differences in MIS protein levels were not detected prior to birth, however, at both 14 and 25 dpp, WC boars had greater levels of MIS compared with MS boars (Fig. 9).

GATA4 protein was present in the nuclei of Sertoli cells within the seminiferous tubules, peritubular cells surrounding the tubules and Leydig cells of the interstitium, but not in germ cells (Fig. 7C). Sertoli cell GATA4 protein levels increased during fetal life to 90 dpc in both breeds, declining thereafter until birth (Fig. 10). Following birth, Sertoli cell GATA4 protein levels increased to 14 dpp in both breeds, declining thereafter (Fig. 10). Sertoli cell GATA4 protein levels did not differ between breeds, but a breed × age interaction indicated a divergence in GATA4 levels in favor of WC boars in postnatal life (Fig. 10).

$P450_{c17}$ protein was present in Leydig cells of the interstitium of the testis in both breeds throughout the

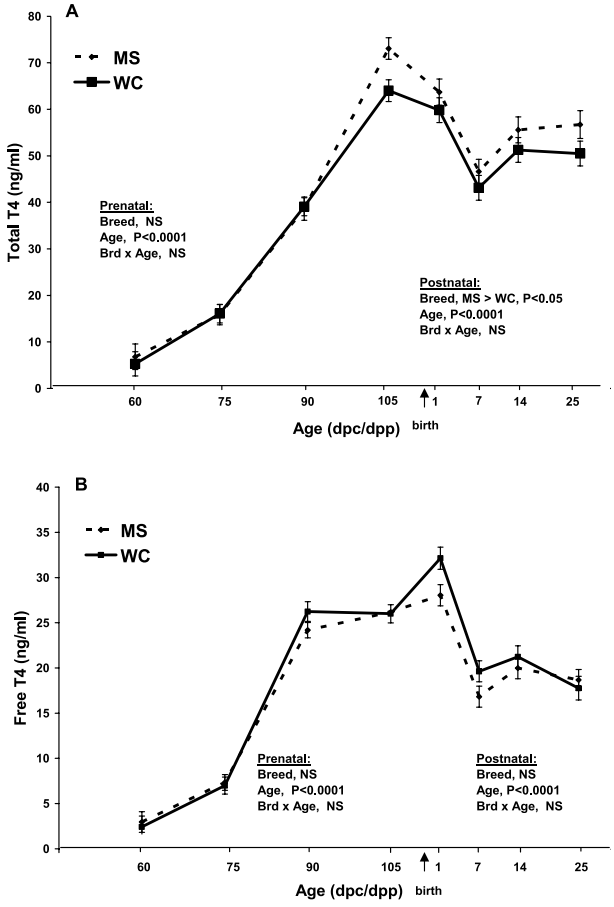

Figure 4 Concentration of total thyroxine (T4) (A) and free T4 (B) in the circulation (ng/ml) of Meishan (MS) and White Composite (WC) boars during fetal and neonatal life. Data are presented as least square means ± S.E. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

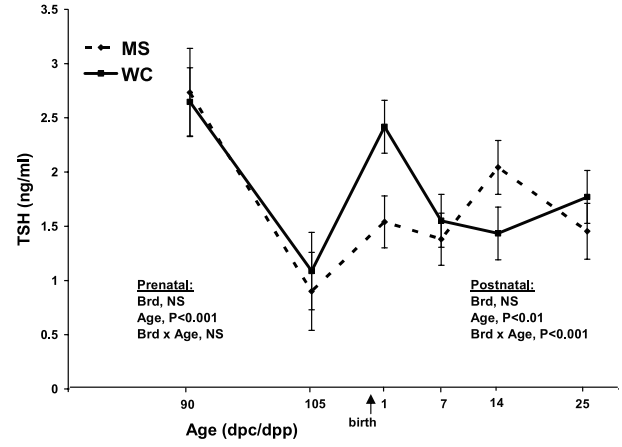

Figure 5 Concentration of thyroid stimulating hormone (TSH) in the circulation (ng/ml) of Meishan (MS) and White Composite (WC) boars during fetal and neonatal life. Data are presented as least square means ± S.E. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

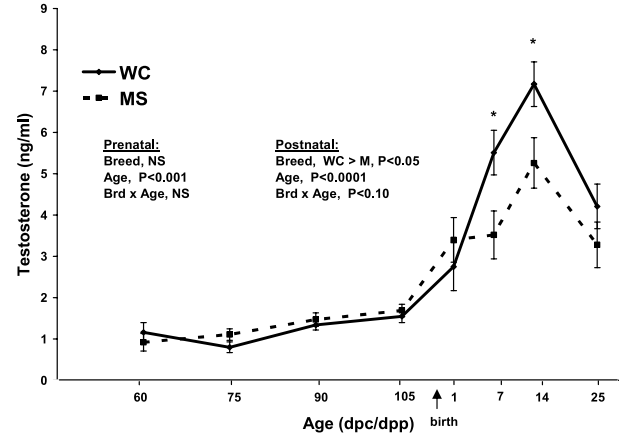

Figure 6 Concentration of testosterone in the circulation (ng/ml) of Meishan (MS) and White Composite (WC) boars during fetal and neonatal life. Data are presented as least square means ± S.E. *$P<0{\cdot}05$. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

period of study (Fig. 7D). $P450_{c17}$ protein levels increased from 75 to 90 dpc declining thereafter until birth in both breeds. Following birth $P450_{c17}$ protein levels were elevated at 7 and 25 dpp but depressed at 14 dpp in both breeds (Fig. 11). WC boars tended to have greater $P450_{c17}$ protein levels compared with MS boars prenatally, but MS boars had greater levels of $P450_{c17}$ protein compared with WC boars, notably at 7 dpp (Fig. 11).

Inhibin-α subunit was present in the cytoplasm of many Leydig cells at 60 and 75 dpc but the number of cells producing inhibin-α decreased thereafter (Fig. 7E). By birth, inhibin-α protein was absent from Leydig cells (Fig. 7F). In contrast, inhibin-α protein was present at high levels in the cytoplasm of Sertoli cells throughout the period of study (Fig. 7E and F). Levels of inhibin-α protein increased from 75 dpc to 7 dpp in both breeds, declined thereafter in MS boars, but continued to increase with age in WC boars (Fig. 12A). Meishan boars had greater levels of inhibin-α at 7 dpp but lower levels by 25 dpp compared with WC boars (Fig. 12A). Inhibin βA subunit was present in the cytoplasm of Leydig and Sertoli cells and at low levels in the cytoplasm of germ cells in both breeds throughout the period of study (Fig. 7G). Sertoli cell inhibin βA decreased from 60 to 90 dpc but increased from 90 to 105 dpc in both breeds (Fig. 12B). From 105 dpc onward, Sertoli cell inhibin βA levels declined in both breeds until 25 dpp (Fig. 12B), but breed differences were not apparent at any stage throughout the study. Inhibin βB subunit protein was present in the cytoplasm and nuclei of both Leydig cells and Sertoli cells but was absent from germ cells in both breeds (Fig. 7H). Sertoli cell inhibin βB protein levels decreased from 60 to 90 dpc in both breeds, followed by an increase until 105 dpc. A second increase at

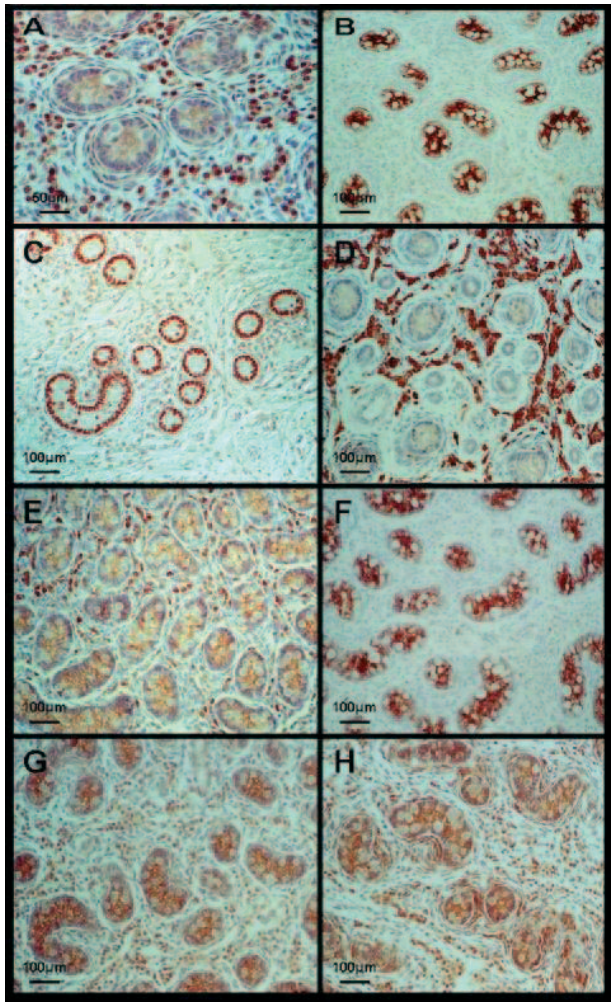

Figure 7 Brightfield photomicrographs of testes immunohistochemically stained with molecular markers. (A) Thyroid hormone receptor β1 localization in a Meishan testis at 90 days post coitum (dpc). (B) Müllerian inhibiting substance localization in a White Composite testis at 105 dpc. (C) GATA4 localization in a White Composite testis at 25 days postpartum (dpp). (D) $P450_{c17}$ localization in a Meishan testis at 105 dpc. (E and F) Inhibin alpha localization in a Meishan testis at 75 dpc (E) and 105 dpc (F). Note the absence of inhibin alpha staining in the interstitium of testes at 105 dpc. (G) Inhibin βA localization in a White Composite testis at 75 dpc. (H) Inhibin βB localization in a White Composite testis at 105 dpc. Red color depicts positive staining. Sections are counterstained blue with hematoxylin to visualize the tissue.

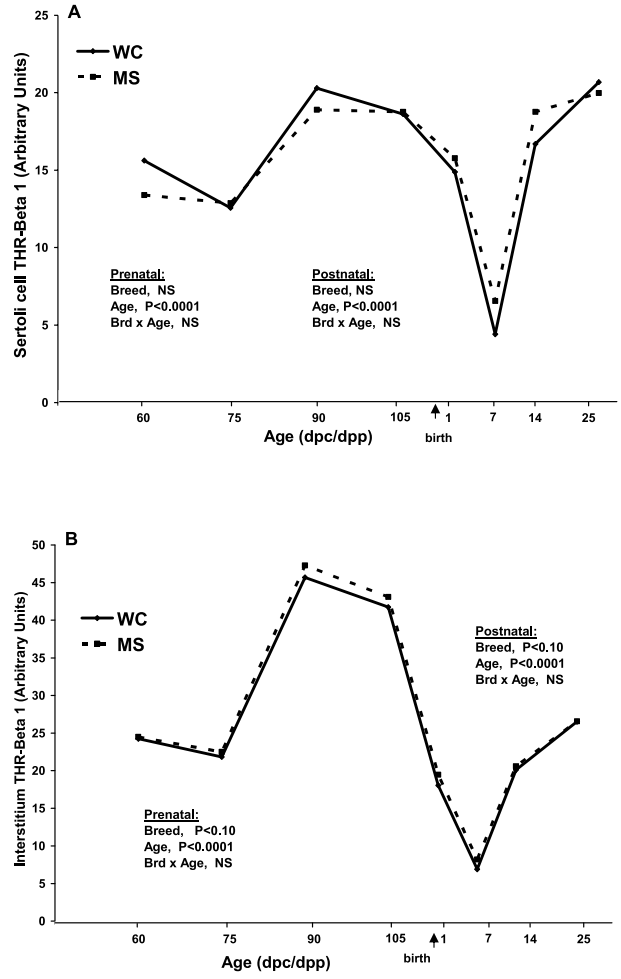

Figure 8 Densitometric values (arbitrary units) for THRβ1 protein levels in the Sertoli cells (A) and Leydig cells (B) of Meishan (MS) and White Composite (WC) testes during fetal and neonatal life. Data are presented as least square means ± S.E. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

14 dpp in both breeds was also observed (Fig. 12C), but breed differences were not apparent.

Discussion

Thyroid hormones play a critical role in regulating the growth, development, differentiation and metabolism of virtually all tissues of higher organisms. In the rodent testis, elevated T_3 inhibits Sertoli cell mitosis, promotes differentiation and accelerates tubular lumen formation (van Haaster *et al.* 1993, Cooke *et al.* 1994) whilst transient neonatal hypothyroidism prolongs the Sertoli cell proliferative period by delaying Sertoli cell maturation, thus leading to increased Sertoli cell number and testicular size (Cooke & Hess 1992, van Haaster *et al.* 1992, Joyce *et al.* 1993, Bunick *et al.* 1994, De Franca *et al.* 1996). However, modification of thyroid hormone levels in rams gives inconsistent results. Induction of hyperthyroidism from 16–24 weeks reduces testis size at 30 weeks of age (Chandrasekhar *et al.* 1985, 1986*a*) whilst hypothyroidism during this time period has no effect on testicular size (Chandrasekhar *et al.* 1985). In contrast, induction of hyperthyroidism from 6–8 weeks increases testis size and

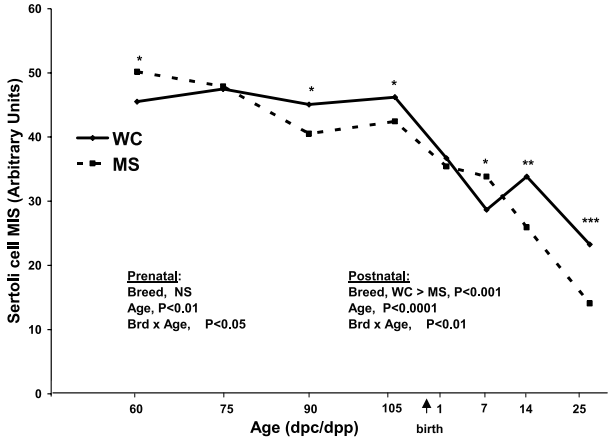

Figure 9 Densitometric values (arbitrary units) for Müllerian inhibiting substance (MIS) protein levels in Sertoli cells of Meishan (MS) and White Composite (WC) testes during fetal and neonatal life. Data are presented as least square means ± S.E. $*P<0{\cdot}05$; $**P<0{\cdot}01$; $***P<0{\cdot}001$. dpc, days post coitum; dpp, days post partum; brd, breed.

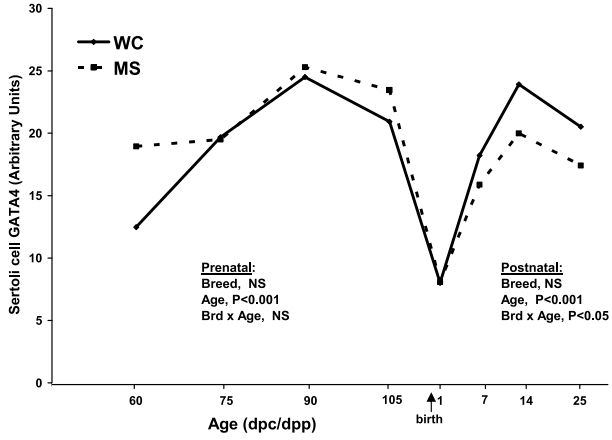

Figure 10 Densitometric values (arbitrary units) of GATA4 protein levels in Sertoli cells of Meishan (MS) and White Composite (WC) testes during fetal and neonatal life. Data are presented as least square means ± S.E. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

advances puberty (Fallah-Rad *et al.* 2001). These observations indicate that the timing of thyroid hormone manipulation in rams may be important, as observed in rodents (Cooke & Hess 1992, van Haaster *et al.* 1992, Joyce *et al.* 1993, Bunick *et al.* 1994, De Franca *et al.* 1996). In cattle, T_3 and T_4 are negatively correlated to testicular volume (Majdic *et al.* 1998). Collectively, these observations support a potential role for thyroid hormones in testicular development.

In pigs, thyroid activity begins by mid gestation (Slebodzinski & Brzezinska-Slebodzinska 1994). During late gestation MS boars are hyperthyroid compared with WC boars, having approximately 30–40% higher levels of T_3, the biologically active thyroid hormone, compared

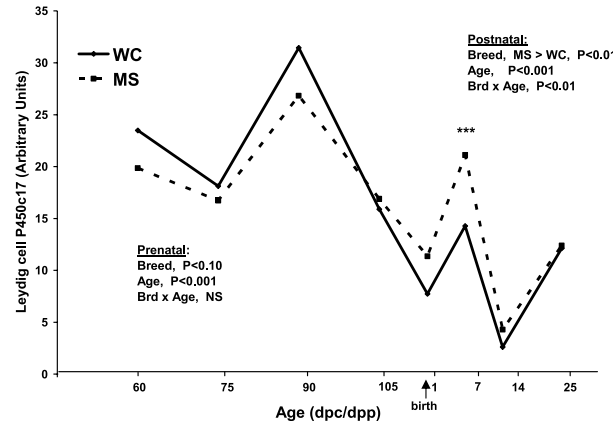

Figure 11 Densitometric values (arbitrary units) of P450c17 protein levels in Leydig cells of Meishan (MS) and White Composite (WC) testes during fetal and neonatal life. Data are presented as least square means ± S.E. $***P<0{\cdot}001$. dpc, days post coitum; dpp, days post partum; brd, breed.

with WC fetuses. Thyroid hormones, in particular T_3, exert their effects by interacting with an intra-nuclear receptor (Samuels & Tsai 1973). The presence of thyroid hormone receptors in a tissue provides an index of thyroid responsiveness (Oppenheimer *et al.* 1974, 1976). Substantial discrepancies exist in the literature regarding the tissue- and cell-specific localization of THR isoforms. It is reported that THRβ1 is absent from the immature testis in rats (Strait *et al.* 1991, Jannini *et al.* 1994, 1999) and humans (Jannini *et al.* 2000) using RNase protection assay, Northern or immunofluorescence analysis. However, Buzzard *et al.* (2000), using immunohistochemistry, reported low levels of THRβ1 mRNA but abundant THRβ1 protein in immature Sertoli cells and in almost all interstitial cells of the neonatal testis in rats. Similarly, Palmero *et al.* (1995) detected THRβ1 in Sertoli cells of both rats and porcine testes using PCR, and Macchia *et al.* (1990) localized THRβ1 to the interstitium and germ cells at the periphery of tubules. These studies indicate that differential sensitivity of detection methods probably account for discrepancies between studies in the tissue-specific expression of THR. Similar discrepancies exist for localization of THRα (Strait *et al.* 1991, Buzzard *et al.* 2000, Jannini *et al.* 2000). The present study is the first to report immunolocalization of THRβ1 in porcine testes, confirming the previous localization of THRβ1 in Sertoli cells using PCR (Palmero *et al.* 1995). Immunodetection of THRβ1 in interstitial cells was unexpected as Palmero *et al.* (1992) failed to detect nuclear binding of T_3 in porcine Leydig cells. During late gestation, levels of THRβ1 correlate better with high affinity binding of thyroid hormone than does THRα1 (Murray *et al.* 1988, Strait *et al.* 1991). Elevated levels of THRβ1 in the porcine testis during this time probably represents a time of maximal responsiveness of the testis to thyroid hormones.

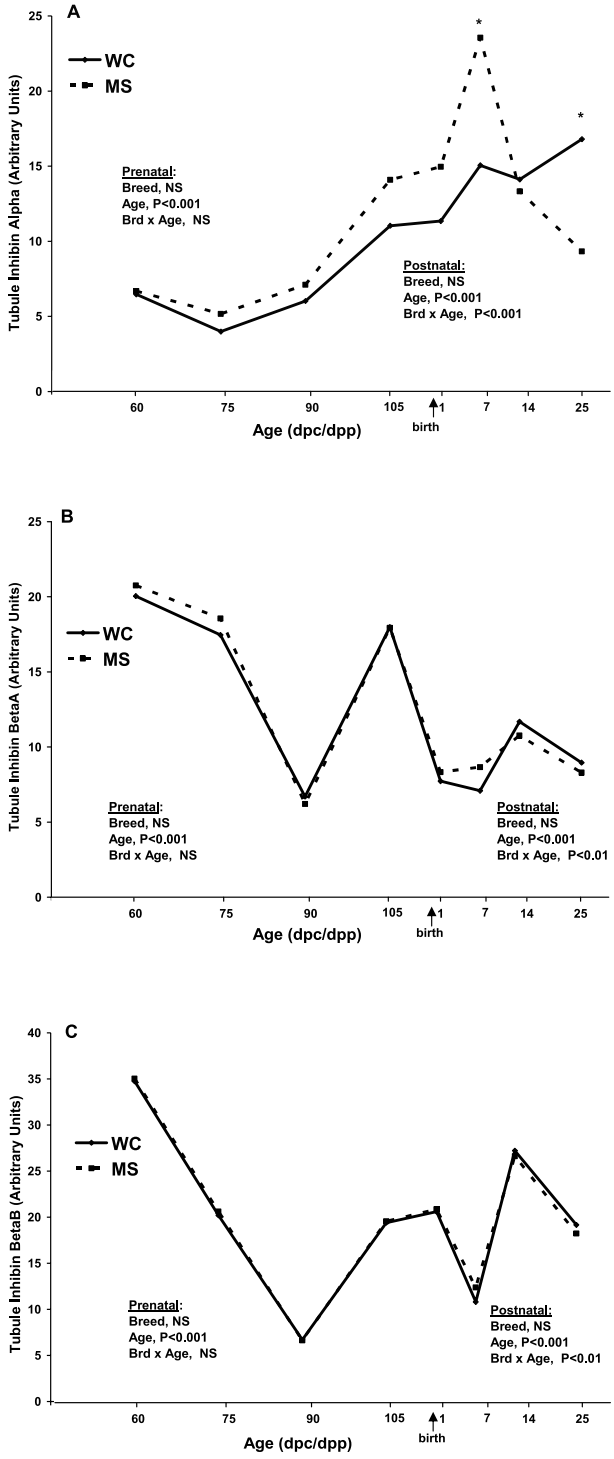

Figure 12 Densitometric values (arbitrary units) of inhibin alpha (A), inhibin βA (B) and inhibin βB (C) protein levels in seminiferous tubules of Meishan (MS) and White Composite (WC) testes during fetal and neonatal life. Data are presented as least square means ± S.E. *$P<0{\cdot}05$. dpc, days post coitum; dpp, days post partum; brd, breed; NS, not significant.

Thus, elevated levels of T_3 during this period of development in MS boars, coupled with increased THRβ1 levels, provides a potential mechanism for 'conditioning' immature Sertoli cells.

Previous studies have highlighted the fact that in order to achieve manipulation of testicular development by thyroid hormone, treatment must be administered during periods of maximal Sertoli cell proliferation (Cooke & Hess 1992, Meisami *et al.* 1992). Late gestation, a time when MS boars are hyperthyroid compared with WC boars, corresponds to the period of maximal Sertoli cell proliferation in the boar (McCoard *et al.* 2003 – companion paper). Sertoli cell proliferation rates peak around 90 dpc in both MS and WC boars (McCoard *et al.* 2003 – companion paper), corresponding to elevated levels of circulating free T_3 and THRβ1, indicating an association between thyroid hormone and Sertoli cell proliferation in the porcine testis. Subsequent reduction in testicular THRβ1 protein levels corresponds with the decline in the rates of Sertoli cell mitosis (McCoard *et al.* 2003 – companion paper), further supporting a link between thyroid hormone and Sertoli cell proliferation in boars. Further, transient fetal hyperthyroidism in MS boars is associated with decreased Sertoli cell proliferation by 14 dpp and enhanced tubule size by 25 dpp, indicative of early maturation of Sertoli cells (McCoard *et al.* 2003 – companion paper).

Down-regulation of MIS, a member of the TGFβ family, is an early sign of testicular maturation in boars (Tran *et al.* 1981) and humans (Baker & Hutson 1993, Rey *et al.* 1993). A decline in MIS protein levels in MS boars during early postnatal life compared with WC boars in the present study is consistent with decreased Sertoli cell proliferation and early maturation of Sertoli cells in this breed. Similarly, elevated MIS protein levels in WC compared with MS boars during neonatal life is consistent with prolonged MIS expression and delayed Sertoli cell maturation following transient neonatal hypothyroidism in rats (Bunick *et al.* 1994). Further, adult MS boars have a reduced complement of Sertoli cells, consistent with a reduced proliferative period, and reach puberty earlier compared with occidental breeds (Lunstra *et al.* 1997), consistent with advanced puberty in rams following transient neonatal hyperthyroidism (Fallah-Rad *et al.* 2001). Collectively, these observations indicate the potential for thyroid hormone to impact on testicular development and onset of puberty in boars.

The substantial increase in T_3 observed around birth is probably due to preparation for the thermal challenge after birth, as newborn pigs lack brown adipose tissue and do not react metabolically to noradrenaline (Le Blanc & Mount 1968), thus relying on thyroid hormone control of thermoregulation (Slebodzinski 1979, 1988). Substantially elevated hepatic and kidney 5′-monodeiodinase type 1 activity, the enzyme that converts T_4 to T_3, during late gestation in the pig supports this physiological event

(Slebodzinski & Brzezinska-Slebodzinska 1994). Whilst a twofold difference in T_3 levels between breeds is apparent from 1 to 7 dpp, T_3 is unlikely to be associated with Sertoli cell proliferation during this time as receptor levels for both breeds are low indicating reduced responsiveness of testicular tissue to thyroid hormone during this time. Sertoli cell proliferation is also declining during this time period (McCoard *et al.* 2003 – companion paper).

TBG is the major transport protein for thyroid hormones and thus has the potential to alter tissue availability of thyroid hormones. TBG is located within the QTL region for testis size on the X-chromosome (Rohrer *et al.* 2001, McCoard *et al.* 2002*a*) and is thus a potential candidate factor for regulation of testicular size. While total T_4 levels were greater in MS than WC boars during early postnatal life, circulating levels of free T_4 were not different between breeds, probably resulting from elevated TBG concentrations (decreased T_3 uptake) in MS compared with WC boars. Similarly, total T_3 concentrations from 90 to 105 dpc are similar, while circulating levels of free T_3 decline from 90 to 105 dpc, a time of elevated TBG concentration. Profiles of T_3 uptake exhibit a similar pattern to Sertoli cell proliferation during fetal life. Elevated T_3 uptake at 90 dpc corresponds to decreased TBG levels, consistent with elevated levels of circulating free T_3 during this phase of development. These observations indicate a potential role for TBG in the regulation of circulating levels of free thyroid hormone, and a possible link between TBG and testicular development in the boar. Potential TBG regulation of site-specific release of thyroid hormone via proteolytic cleavage of TBG (Schussler 2000) cannot be discounted. Since more than two thirds of the circulating thyroid hormone is bound by TBG, proteolytic cleavage of TBG could potentially allow substantially greater site-specific release of thyroid hormone than is available from free thyroid hormone. Thus, increased TBG in MS boars during late fetal life provides a potential mechanism to increase thyroid hormone availability to the testis. Further studies will be required to test these hypotheses.

A wide array of molecular markers is expressed during testicular development. Modification of testicular development, such as by induced hypothyroidism (Bunick *et al.* 1994) can alter expression patterns of these markers. GATA4 is a member of the GATA family of transcription factors expressed in the gonads (Heikinheimo *et al.* 1997, Viger *et al.* 1998, Ketola *et al.* 1999, McCoard *et al.* 2001*a*,*b*), and plays an important role in the transcriptional activation of numerous target genes (Tremblay & Viger 2001) including MIS (Viger *et al.* 1998, Tremblay & Viger 1999), inhibin βB (Feng *et al.* 2000), inhibin-α (Ketola *et al.* 1999, Feng *et al.* 1998) and the steroidogenic acute regulatory protein (StAR) (Silverman *et al.* 1999). In the present study, GATA4 protein levels were upregulated in Sertoli cells of the boar testis during the period of maximal Sertoli cell proliferation (~90 dpc), consistent with observations in the human testis (Ketola *et al.* 2000) indicating a potential role in Sertoli cell development in the boar. GATA4 protein levels also exhibit similar patterns compared with THRβ1 levels (tubules and interstitium) indicating a potential relationship between these two molecular markers during testicular development.

Inhibin produced by the testis regulates FSH secretion from the pituitary. Both inhibin and activin have been reported to influence cell proliferation, apoptosis and differentiation in many systems (de Jong 1988). Inhibin subunits can act as paracrine factors in the gonads and are expressed during fetal life in many species including humans (Eramma *et al.* 1992, Roberts 1997), rodents (Roberts *et al.* 1989, Shaha *et al.* 1989, Roberts & Barth 1994), monkeys (Rabinovici *et al.* 1991), sheep (Jarred *et al.* 1999), and cattle (Torney *et al.* 1990). Until now, testicular localization of inhibin subunits in boars was uncharacterized. Consistent with observations in the fetal sheep testis (Jarred *et al.* 1999), Sertoli cells in the boar testis produce all inhibin subunits during fetal and early postnatal life indicating the potential to produce all forms of inhibin and activin. In contrast, down-regulation of inhibin-α in Leydig cells during fetal development indicates that while Leydig cells can produce both inhibins and activins during fetal life, they are unable to synthesize inhibins during postnatal development. Inhibin subunit levels do not correlate well with circulating FSH (McCoard *et al.* 2003 – companion paper), or patterns of Sertoli cell proliferation, further indicating that elevated gonadotropins are unlikely regulators of Sertoli cell proliferation in boars. However, Sertoli cell inhibin-α subunit levels decline from 7 dpp onward in MS boars, corresponding to early Sertoli cell maturation in this breed of pig, suggesting a potential paracrine role for inhibin-α in the boar testis.

During neonatal life, WC boars exhibit a greater peak in testosterone secretion at 14 dpp compared with MS boars, consistent with other studies (Lunstra *et al.* 1997, Franca *et al.* 2000). Breed differences in testosterone secretion are associated with substantial breed differences in interstitial tissue growth during this time period (McCoard *et al.* 2003 - companion paper). In contrast, Leydig cell $P450_{c17}$ protein levels, the enzyme that catalyzes conversion of progesterone to androstenedione, are greater in MS than WC boar testis during neonatal life, indicating differential enzymatic steroidogenic activity of the testis. The second wave of Leydig cell development occurs from approximately 75 dpc to 1 month of age in boars (van Vorstenbosch *et al.* 1984). Thus, differential levels of $P450_{c17}$ probably indicate enhanced Leydig cell development during early neonatal life in MS boars, perhaps in preparation for early onset of puberty in this breed (Lunstra *et al.* 1997).

In summary, transient hyperthyroidism in MS boars during mid to late gestation corresponds to the stage of development when Sertoli cell proliferation is maximal

and the testis is highly responsive to thyroid hormone, providing a mechanism for thyroid hormones to impact Sertoli cell development. Subsequent decline in proliferation rate and early down-regulation of MIS expression, coupled with increased seminiferous tubule diameter, signal early maturation of Sertoli cells, consistent with early onset of puberty in this breed. These observations indicate a possible role for thyroid hormones in the regulation of growth and differentiation of the boar testis via direct action on Sertoli cells. We cannot rule out the possibilty that differences in thyroid status may, in part, be correlated with differences in the rate of sexual maturity, body composition etc. that exists between these diverse genetic lines of pigs (Stone *et al.* 1985, Herpin *et al.* 1993, White *et al.* 1995).

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Names are necessary to report factually on the available data; however the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

References

Baker ML & Hutson JM 1993 Serum levels of Müllerian inhibiting substance in boys throughout puberty and in the first two years of life. *Journal of Clinical Endocrinology and Metabolism* **76** 245–247.

Bunick D, Kirby J, Hess RA & Cooke PS 1994 Developmental expression of testis messenger ribonucleic acids in the rat following propylthiouracil-induced neonatal hypothyroidism. *Biology of Reproduction* **51** 706–713.

Buzzard JJ, Morrison JR, O'Bryan MK, Song Q & Wreford NG 2000 Developmental expression of thyroid hormone receptors in the rat testis. *Biology of Reproduction* **62** 664–669.

Chandrasekhar Y, D'Occhio MJ, Holland MK & Setchell BP 1985 Activity of the hypothalamo–pituitary axis and testicular development in prepubertal ram lambs with induced hypothyroidism or hyperthyroidism. *Endocrinology* **117** 1645–1651.

Chandrasekhar Y, D'Occhio MJ & Setchell BP 1986*a* Delayed puberty caused by hyperthyroidism in ram lambs is not a result of suppression in body growth. *Journal of Reproduction and Fertility* **76** 763–769.

Chandrasekhar Y, D'Occhio MJ & Setchell BP 1986*b* Reproductive hormone secretion and spermatogenic function in thyroidectomized rams receiving graded doses of exogenous thyroxine. *Journal of Endocrinology* **111** 245–253.

Cooke PS & Hess RA 1992 The pattern of Sertoli cell proliferation is altered in the neonatal hypothyroidism model for increased testis size. *Journal of Andrology* **13** (Suppl) Abstract 47.

Cooke PS, Zhao YD & Bunick D 1994 Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. *Biology of Reproduction* **51** 1000–1005.

Davies AG 1971 Histological changes in the seminiferous tubules of immature mice following administration of gonadotrophins. *Journal of Reproduction and Fertility* **25** 21–28.

De Franca LR, Hess RA, Cooke PS & Russell LD 1996 Neonatal hypothyroidism causes delayed Sertoli cell maturation in rats treated with propylthiouracil: evidence that the Sertoli cell controls testis growth. *Anatomical Record* **242** 57–69.

Eramma M, Keikinheimo K & Voutilainen R 1992 Developmental and cyclic adenosine 3′, 5′ monophosphate-dependent regulation of inhibin subunit messenger ribonucleic acids in human fetal testes. *Journal of Clinical Endocrinology and Metabolism* **75** 806–811.

Fallah-Rad AH, Connor ML & Del Vecchio RP 2001 Effect of transient early hyperthyroidism on onset of puberty in Suffolk ram lambs. *Reproduction* **121** 639–646.

FASS 1999 *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. 1st rev. Ed. Savoy, IL: Federation of Animal Science Societies.

Feng ZM, Wu AZ & Chen CL 1998 Testicular GATA-1 factor regulates the promoter activity of rat inhibin α-subunit gene in MA-10 Leydig tumor cells. *Molecular Endocrinology* **12** 378–390.

Feng ZM, Wu AZ, Zhing Z & Chen CL 2000 GATA-1 and GATA-4 transactivate inhibin/activin beta-B-subunit gene transcription in testicular cells. *Molecular Endocrinology* **14** 1820–1835.

Ford JJ, Wise TH, Lunstra DD & Rohrer GA 2001 Interrelationships of porcine X and Y chromosomes with pituitary gonadotropins and testicular size. *Biology of Reproduction* **65** 906–912.

Franca LR, Silva VA Jr, Chiarini-Garcia H, Garcia SK & Debeljuk 2000 Cell proliferation and hormonal changes during postanatal development in the pig. *Biology of Reproduction* **63** 1629–1636.

Gondos B & Berndtson WE 1993 Postnatal and pubertal development. In *The Sertoli Cell*, pp 116–153. Eds LD Russell & MD Griswold. Clearwater, FL: Cache River Press.

Griswold MD 1993 Action of FSH on mammalian Sertoli cells. In *The Sertoli Cell*, pp 788–792. Eds LD Russell & Griswold MD. Clearwater, FL: Cache River Press.

Griswold MD, Solari A, Tung PS & Irving B 1977 Stimulation by follicle-stimulating hormone of DNA synthesis and of mitosis in cultured Sertoli cells prepared from the testis of immature rats. *Molecular and Cellular Endocrinology* **7** 151–165.

van Haaster LH, de Jong FH, Docter R & de Rooij DG 1992 The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. *Endocrinology* **131** 1574–1576.

van Haaster LH, de Jong FH, Docter R & de Rooij DG 1993 High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. *Endocrinology* **133** 755–760.

Heikinheimo M, Ermolaeva M, Bielinska M, Rahman RA, Narita N, Huhtaniemi IT, Tapanainin JS & Wilson DB 1997 Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* **138** 3505–3514.

Herpin P, Dividich JL & Amaral N 1993 Effect of selection for lean tissue growth on body composition and physiological state of the pig at birth. *Journal of Animal Science* **71** 2645–2653.

Jannini EA, Dolci S, Ulisse S & Nikodem VM 1994 Developmental regulation of the thyroid hormone receptor α1 mRNA expression in the rat testis. *Molecular Endocrinology* **8** 89–96.

Jannini EA, Carosa E, Rucci N, Screponi E & Armiento MD 1999 Ontogeny and regulation of variant hormone receptor isoforms in developing rat testis. *Journal of Endocrinological Investigation* **22** 843–848.

Jannini EA, Crescenzi A, Rucci N, Screponi E, Carosa E, De Matteis A, Macchia E, D'Amati G & D'Armiento M 2000 Ontogenetic

pattern of thyroid hormone receptor expression in the human testis. *Journal of Clinical Endocrinology and Metabolism* **85** 3453–3457.

Jarred RA, Cancilla B, Richards M, Groome NP, McNatty KP & Risbridger GP 1999 Differential localization of inhibin subunit proteins in the ovine testis during fetal gonadal development. *Endocrinology* **140** 979–986.

de Jong FH 1988 Inhibin. *Physiological Reviews* **68** 555–607.

Joyce KL, Porcelli J & Cooke PS 1993 Neonatal goitrogen treatment increases adult testis size and sperm production in the mouse. *Journal of Andrology* **14** 448–455.

Ketola I, Rahman N, Toppari J, Bielinska M, Porter-Tinge SB, Tapanainen JS, Huhtaniemi IT, Wilson DB & Heikinheimo M 1999 Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. *Endocrinology* **140** 1470–1480.

Ketola I, Pentikainen V, Vaskivuo T, Ilvesmaki V, Herva R, Dunkel L, Tapanainen JS, Toppari J & Heikinheimo M 2000 Expression of transcription factor GATA-4 during human testicular development and disease. *Journal of Clinical Endocrinology and Metabolism* **85** 3925–3931.

Le Blanc J & Mount LE 1968 Effects of noradrenaline and adrenaline on oxygen consumption rate and arterial blood pressure in the newborn pig. *Nature* **217** 77–78.

Li MD, Matteri RL, Macdonald GJ, Wise TH & Ford JJ 1996 Overexpression of α/β-subunit of thyroid-stimulating hormone in Meishan swine identified by differential display. *Journal of Animal Science* **74** 2104–2111.

Lunstra DD, Ford JJ, Klindt J & Wise TH 1997 Physiology of the Meishan boar. *Journal of Reproduction and Fertility* **52** 181–193.

Lunstra DD, Wise TH & Ford JJ 2003 Sertoli cells in the boar testis: changes during development and compensatory hypertrophy after hemicastration at different ages. *Biology of Reproduction* **68** 140–150.

Macchia E, Nakai A, Janiga A, Sakurai A, Fisfalen ME, Gardner P, Soltani K & DeGroot LJ 1990 Characterization of site-specific polyclonal antibodies to c-erbA peptides recognizing human thyroid hormone receptors α_1, α_2, and β and native 3,5,3′-triiodothyronine receptor, and study of tissue distribution of the antigen. *Endocrinology* **126** 3232–3239.

McCoard SA, Lunstra DD, Wise TH & Ford JJ 2001*a* Specific staining of Sertoli cell nuclei and evaluation of sertoli cell number and proliferative activity in Meishan and White Composite boars during the neonatal period. *Biology of Reproduction* **64** 689–695.

McCoard SA, Wise TH, Fahrenkrug SC & Ford JJ 2001*b* Temporal and spatial localization patterns of GATA4 during porcine gonadogenesis. *Biology of Reproduction* **65** 366–374.

McCoard SA, Fahrenkrug SC, Alexander LJ, Freking BA, Rohrer GA, Wise TH & Ford JJ 2002*a* An integrated comparative map of the porcine X chromosome. *Animal Genetics* **33** 178–185.

McCoard SA, Wise TH & Ford JJ 2002*b* Expression levels of Müllerian-inhibiting substance, GATA4 and 17α-hydroxylase/17,20-lyase cytochrome P450 during embryonic gonadal development in two diverse breeds of swine. *Journal of Endocrinology* **175** 365–374.

McCoard SA, Wise TH, Lunstra DD & Ford JJ 2003 Stereological evaluation of Sertoli cell ontogeny during fetal and neonatal life in two diverse breeds of swine. *Journal of Endocrinology* **178** 395–403 (see this issue).

Majdic G, Snoj T, Horvat A, Mrkun J, Kosec M & Cestnik V 1998 Higher thyroid hormone levels in neonatal life result in reduced testis volume in postpubertal bulls. *International Journal of Andrology* **21** 352–357.

Marshall GR & Plant TM 1996 Puberty occurring either spontaneously or induced precociously in Rhesus monkey (*Macaca mulatta*) is associated with a marked proliferation of Sertoli cells. *Biology of Reproduction* **54** 1192–1199.

Meachem SJ, McLachlan RI, de Krester DM, Robertson DM & Wreford NG 1996 Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. *Biology of Reproduction* **54** 36–44.

Meisami E, Sendera TJ & Clay LB 1992 Paradoxical hypertrophy and plasticity of the testis in rats recovering from early thyroid deficiency: a growth study including effects of age and duration of hypothyroidism. *Journal of Endocrinology* **135** 495–505.

Murray MB, Silz ND, McCreary NL, MacDonald MJ & Towle HC 1988 Isolation and characterization of rat cDNA clones for two distinct thyroid hormone receptors. *Journal of Biological Chemistry* **263** 12770–12777.

Oppenheimer JH, Schwartz HL & Surks MI 1974 Tissue differences in the concentration of triiodothyronine nuclear binding sites in the rat: liver, kidney, pituitary, heart, brain, spleen and testis. *Endocrinology* **95** 897–903.

Oppenheimer JH, Schwartz HL, Surks MI, Koerner DH & Dillman WH 1976 Nuclear receptors and the initiation of thyroid hormone action. In *Recent Progress in Hormone Research*, vol 32, pp 529–565. Ed RO Greep. Orlando, FL: Academic Press.

Orth JM 1984 The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in the testes of fetal rats. *Endocrinology* **115** 1248–1255.

Palmero S, Benahmed M, Morera AM, Trucchi P & Fugassa E 1992 Identification of nuclear tri-iodothyronine receptors in Sertoli cells from immature piglet testes. *Journal of Molecular Endocrinology* **9** 55–59.

Palmero S, De Marco P & Fugassa E 1995 Thyroid hormone receptor β mRNA expression in Sertoli cells isolated from prepubertal testis. *Journal of Molecular Endocrinology* **14** 131–134.

Pelliniemi LJ, Frojdman K & Paranko J 1993 Embryological and postnatal development and function of Sertoli cells. In *The Sertoli Cell*, pp 87–114. Eds LD Russell & MD Griswold. Clearwater FL: Cache River Press.

Rabinovici J, Goldsmith PC, Roberts VJ, Vaughan J, Vale W & Jaffe RB 1991 Localization and secretion of inhibin/activin subunits in the human and sub-human primate fetal gonads. *Journal of Clinical Endocrinology and Metabolism* **73** 1141–1149.

Rey R, Lordereau-Ruchard I, Carel JC, Barbet P, Cate RL, Roger M, Chaussain JL & Josso N 1993 Anti-Müllerian hormone and testosterone serum levels are inversely related during normal and precocious pubertal development. *Journal of Clinical Endocrinology and Metabolism* **77** 1220–1226.

Roberts VJ 1997 Tissue-specific expression of inhibin/activin subunit and follistatin mRNAs in mid- to late-gesational age human fetal testis and epididymis. *Endocrine* **6** 85–90.

Roberts VJ & Barth SL 1994 Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid- to late-gestation rat embryogenesis. *Endocrinology* **134** 914–923.

Roberts V, Meunier H, Sawchenki PE & Vale W 1989 Differential production and regulation of inhibin subunits in rat testicular cell types. *Endocrinology* **125** 2350–2359.

Rohrer GA, Wise TH, Lunstra DD & Ford JJ 2001 Identification of genomic regions controlling plasma FSH concentrations in Meishan-White composite boars. *Physiological Genomics* **6** 145–151.

Samuels HH & Tsai JS 1973 Thyroid hormone action in cell culture: demonstration of nuclear receptors in intact cells and isolated nuclei. *PNAS* **70** 3488–3492.

SAS 1999 SAS *User's Guide: Statistics*. 8th Ed. SAS Cary, NC: Institute Inc.

Schussler, GC 2000 The thyroxine-binding proteins. *Thyroid* **10** 141–149.

Shaha C, Morris PL, Chem CL, Vale W & Bardin CW 1989 Immunostainable inhibin subunits are in multiple types of testicular cells. *Endocrinology* **125** 1941–1950.

Silverman E, Eimerl S & Orly J 1999 CCAAT enhancer-binding protein beta and GATA-4 binding regions within the promoter of the steroidogenic acute regulatory protein (StAR) gene are required for transcription in rat ovarian cells. *Journal of Biological Chemistry* **274** 17987–17996.

Slebodzinski AB 1979 Metabolic response to thyroxine in the newborn pig. *Biology of the Neonate* **36** 198–205.

Slebodzinski AB 1988 Hyperiodothyroninaemia of neonates, its significance for thermogenesis. *Acta Physiologica Polonica* **39** 364–379.

Slebodzinski AB & Brzezinska-Slebodzinska E 1994 The appearance and activity of tissue thyroxine 5′- and 5-monodeiodinase during ontogenesis in the fetal pig. *Journal of Endocrinology* **141** 243–249.

Strait KA, Schwartz HL, Seybold VS, Ling NC & Oppenheimer JH 1991 Immunofluorescence localization of thyroid hormone receptor protein β1 and variant α2 in selected tissues: cerebellar Purkinje cells as a model for β1 receptor-mediated developmental effects of thyroid hormone in the brain. *PNAS* **88** 3887–3891.

Stone RT, Campion DR, Klindt J & Martin RJ 1985 Blood parameters and body composition in fetuses from reciprocal crosses of genetically lean and obsese swine. *Proceedings of the Society for Experimental Biology and Medicine* **180** 191–195.

Tarn CY, Rosenkrans CF Jr, Apple JK & Kirby JD 1998 Effect of 6-N-propyl-2-thiouracil on growth, hormonal profiles, carcass and reproductive traits of boars. *Animal Reproduction Science* **50** 81–94.

Torney AH, Robertson DM, Hodgson YM & de Kretser DM 1990 *In vitro* bioactive and immunoactive inhibin concentrations in bovine fetal ovaries and testes throughout gestation. *Endocrinology* **127** 2938–2946.

Tran D, Muesy-Dessolle N & Josso N 1981 Waning of anti-müllerian activity: an early sign of Sertoli cell maturation in the developing pig. *Biology of Reproduction* **24** 923–931.

Tremblay JJ & Viger RS 1999 Transcription factor GATA-4 enhances Müllerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. *Molecular Endocrinology* **13** 1388–1401.

Tremblay JJ & Viger RS 2001 GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. *Endocrinology* **142** 974–976.

van Vorstenbosch CJ, Colenbrander B & Wensing CJ 1984 Leydig cell development in the pig testis during the late fetal and early postnatal period: an electron microscopic study with attention to the influence of fetal decapitation. *American Journal of Anatomy* **169** 121–136.

Viger RS, Mertineit C, Trasler JM & Nemer M 1998 Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Müllerian inhibiting substance promoter. *Development* **125** 2665–2675.

White BR, Lan YH, McKeith FK, Novakofski J, Wheeler MB & McLaren DG 1995 Growth and body composition of Meishan and Yorkshire barrows and gilts. *Journal of Animal Science* **73** 738–749.

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